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(54) Title: BIOLUMINESCENCE AND ASSAY REAGENTS

(57) Abstract

A kit of two more containers, suitable for use in the estimation of ATP released from microorganisms, contains luciferin, luciferase, a reconstitution medium and, if desired, a surfactant or other cell lytic agent, and additionally contains a medium-chain alkanoic acid.

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BIOLUMINESCENCE AND ASSAY REAGENTS

This invention relates to a method for detecting microorganisms.

Live microorganisms can be detected with great sensitivity by measuring their ATP using a reagent based on the firefly luciferase reaction. This ATP must be extracted by lysing the microorganisms. There are presently four main ways of doing this, i.e. extracting with boiling buffer or with detergent, or using solvents or acids.

The detergent method is understandably the most popular because it involves a minimum of sample handling, is almost instantaneous, and allows a simple two-step assay, comprising: (1) adding an extractant to the sample; and (2) adding reagent and measuring light. This method is the subject of GB-A-1604249.

The detergent often actually stimulates the initial light production, but also rapidly inactivates the enzyme. Thus the light output falls to nothing over a period of a few minutes. Therefore, despite the initial stimulation, there may be a drastic loss of assay sensitivity, especially if the light output is not measured straight away.

It is known that non-ionic detergents protect, at least in part, from this inactivation. However, they may also protect bacteria from the lytic effects of the cationic detergent; therefore, a "mixed" detergent extractant is not efficient. Furthermore, non-ionic detergents themselves have a slow inactivating effect on luciferase and cannot be included as part of a stable reagent.

The only way to use a non-ionic detergent as a protectant is to add it between the additions of extractant and reagent. The assay now requires three steps, and is therefore less convenient and more expensive to automate.

An object behind the present invention is to provide a reagent having a low decay rate, i.e. <5% per minute,

e.g. even after the addition of a detergent-based extractant, so that it can be used in a two-step assay. This could be used in even the simplest luminometer, e.g. a camera-type microtitre plate system, and would bring all the advantages of improved accuracy and sensitivity of a true low-decay rate assay.

Surprisingly, it has been found that medium chain-length fatty acids, e.g. C_{5-15} alkanoic acids, are efficient luciferase protectants. Their esters or other derivatives/analogues may also be used. For example, a reagent containing octanoic acid retains low-decay rate kinetics even in the presence of an efficient extractant based on cationic detergents. It has little effect on the storage stability of luciferase and can even be added to a luciferase-luciferin reagent before it is freeze-dried, although the, say, octanoic acid is preferably included in the reagent reconstitution buffer. Such a reagent could still be used after 8 hours at 25°C or 1 week at 4°C.

The following Examples illustrate the invention.

Example 1

Vials of a commercially-available freeze-dried reagent preparation containing firefly luciferase, D-luciferin and stabilisers, a low decay rate cocktail, were dissolved in 5 ml of buffer without and with added octanoic acid. The latter comprised 50 mM Tris-Hepes, 9 mM magnesium acetate, 1 mM EDTA, 1 g/l octanoic acid and 0.02% sodium azide. After incubation at 15°C for 1 hour, the two preparations were compared in an assay of ATP in the presence of a bacterial lysing reagent containing cationic detergents.

The assay was performed as follows using a Berthold Biolumat LB9500T luminometer: 0.1 ml of the lysing agent was placed in a cuvette, 0.1 ml of 2 x 10^{-9} M Na_2ATP in sterile water was added, and the cuvette was equilibrated at 25°C for 5 minutes. 0.1 ml of the reagent was then added. The initial light output was measured by a 10 second integration, and the light signal was continuously monitored on a chart recorded so that the decay rate could

be calculated. The stability of the reconstituted reagents was compared by storage at 25°C and 4°C. The results were as follows:

	Without octanoic acid	With octanoic acid
Response to ATP (RLU/attomol)	0.62	0.42
Decay rate (% min^{-1})	27	<3
Stability 24 hr 25°C (%)	87	60
Stability 1 week 4°C (%)	84	76

These results show that the presence of octanoic acid provides adequate stability in solution for most applications.

Example 2

Vials of a high sensitivity cocktail were reconstituted and assayed as above, except that the concentration of the Na_2ATP solution was 2×10^{-10} M. The decay of the light signal was slower in the presence of octanoic acid, and displayed single exponential kinetics. By determining the half-life of decay and multiplying by the initial light output, an accurate calculation of the total light emitted by the reaction mixture (in arbitrary units) could be made. This was shown to be a more accurate measure of the quantity of ATP than the initial light output alone.

CLAIMS

1. A kit of two more containers, suitable for use in the estimation of ATP released from microorganisms, containing luciferin, luciferase, a reconstitution medium and, if desired, a surfactant or other cell lytic agent, which additionally contains a medium-chain alkanoic acid.

2. A kit according to claim 1, wherein one container contains the medium and the acid.

3. A kit according to claim 1 or claim 2, wherein the acid is octanoic acid.

4. A kit according to any preceding claim, wherein one container contains a freeze-dried preparation including luciferin and luciferase.

5. A kit according to any preceding claim, wherein the medium is a buffer having substantially the optimum pH for the luciferin-luciferase reaction.

6. Any one of the containers defined in any of claims 1 to 5 that contains the acid.

7. An assay of cells, which comprises lysing the cells and measuring the ATP released by means of bioluminescence in the presence of a medium-chain alkanoic acid.

INTERNATIONAL SEARCH REPORT

In' ·onal Application No
PCT/GB 95/02085

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/66

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 268, no. 33, 25 November 1993 MD US, pages 24734-24741, W. A. FRANCISCO ET AL. 'Interaction of bacterial luciferase with aldehyde substrates and inhibitors.' see the whole document --- -/--	1-7

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Authorized officer Griffith, G

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Int. onal Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 110, no. 15, 10 April 1989 Columbus, Ohio, US; abstract no. 131899, D. M. BYERS. 'Elongation of exogenous fatty acids by the bioluminescent bacterium Vibrio harveyi.' page 402; column 1; see abstract & J. BACTERIOL., vol. 171, no. 1, pages 59-64, ---	1-7
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 95/02085

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		SE-A-	8703675	24-03-89
		WO-A-	8902929	06-04-89
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